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Two B cell factors bind the HLA-DRA X box region and recognize different subsets of HLA class II promoters

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ABSTRACT

The class II genes of the human Major Histocompatibility Complex (MHC) encode three isotypes of α/β heterodimeric proteins, HLA-DR, -DQ, and -DP, which are responsible for presenting processed antigens to T helper lymphocytes. These MHC class II genes are expressed in a coordinate manner. The promoter regions of all MHC class II genes share a set of highly conserved elements that mediate different levels of tissue-specific and inducible transcription. One element, the X box, appears to be the major positive element in B cell-specific expression, and nuclear protein binding studies have subdivided this region into the X1 and X2 boxes. Regulatory Factor X (RFX) binds to the X1 box whereas several other factors have been described that bind to the X2 box. In this report, we further characterize the X1 binding protein RFX and show that RFX binds poorly to β chain gene promoters. In particular, RFX does not bind to the DRB gene, which is expressed at the highest levels of all β chain genes. In addition, we have identified an X2 box binding activity in human B cell extracts that binds with high affinity to the HLA-DRA promoter. This X2 binding protein, X2BP, binds to a different subset of class II promoters than does RFX. These findings suggest that coordinate regulation of class II expression may involve different combinations or arrangements of transcriptional elements and factors instead of a common set.

INTRODUCTION

The class II genes of the human Major Histocompatibility Complex (MHC) encode heterodimeric cell surface glycoproteins that present processed antigens to helper T cells. These interactions are central to the initiation of a specific acquired immune response and to the education of immature T lymphocytes (1,2). In humans, three isotypes of class II MHC proteins are encoded in the HLA-DR, -DQ, and -DP subregions. Each subregion contains separate genes for α and β chains of the heterodimer. Temporally, all three isotypes are expressed in a coordinate manner; however, members of each subgroup are transcribed at different levels relative to one another (3). Class II expression exhibits tissue-specificity as it is tightly restricted to a few cell types of the immune system including: mature B lymphocytes, activated T cells, macrophages, dendritic cells, and thymic epithelial cells. In addition, the cytokine γ-interferon (γIFN) can induce class II genes on macrophages, as well as a number of non-immune system cells like fibroblasts and endothelial cells (4).

DNA transfection studies have shown that 160 bp of DNA sequence upstream of the HLA-DRA and HLA-DQB transcriptional start sites are necessary and sufficient for both constitutive and inducible expression (5–7). Within this region are three highly conserved sequence elements, designated the W (or Z), X, and Y boxes. Of the three, the X box region has been shown to be most important for B cell-specific expression, being both necessary and sufficient for class II transcription in these cells (7,8). This element has been further subdivided into the X1 and X2 boxes based upon nuclear protein binding studies. Linker-scanning mutagenesis analysis of the murine I-Aα and I-Eα promoters showed that both X1 and X2 boxes were important for optimal expression in B cells (9,10). The X2 box shows sequence similarity to both the TPA response element (TRE) and the cyclic AMP response element (CRE) (11,12).

The functions of the homologous cis-acting regulatory elements appear to be common to the class II genes that have been studied. However, whether or not a common set of trans-acting factors interacts with the slightly different elements in each of the class II genes is still not resolved. An array of sequence-specific DNA binding proteins have been identified that interact with the W, X1, X2, and Y elements found in the promoters of different class II genes (Reviewed in (13)). Several factors have been identified in human B cells that bind X box sequences. One of these factors, RFX, binds to the X1 box of the HLA-DRA gene (approximately −112 to −98 bp upstream of the transcriptional start site) (14,15). Among α chain gene X boxes, RFX shows the greatest relative binding affinity for DRA, a lowered affinity for DPA, and very weak binding activity to DQA (16). Moreover, RFX binding activity to DRA is absent in B-cell nuclear extracts prepared from patients suffering from class II-deficient combined

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immunodeficiency (CID) or the bare lymphocyte syndrome (BLS) (17). Several human factors that interact with the X2 box have been identified. The factor hXBP-1 binds to the X2 box of DRA and DPB but not to other class II X2 boxes (18). *In vitro*, recombinant hXBP-1 can form heterodimers with c-Fos via a leucine zipper motif (19). The factor NFS (16), which recognizes the sequences adjacent to the 3′ side of the X2 box in the DQA gene, binds weakly to DRA. In addition, it was shown that recombinant c-Jun can also bind to the X2 box of the DRA gene (12). However, detectable levels of this c-Jun binding activity in B cells were not found (12).

In this report, we compare the binding activities of B-cell nuclear proteins to the X1 and X2 boxes of all functional human class II genes. From a Raji cell nuclear extract, we show the formation of two different DNA/protein complexes with the X1 and X2 boxes of the DRA gene: RFX binding to the X1 box and an X2 binding factor, designated here as X2BP. Both factors show differential binding affinities to the various class II promoter elements. RFX binds poorly to all β chain gene X1 box sequences, including that of the DRB gene, which is expressed at the highest levels of all the β chain genes. X2BP, however, binds to the X2 boxes of the DRA, DRB, and DPB genes but not those of the DQA, DQB, and DPA genes. In contrast to what was expected for homologous genes and promoter elements, these results suggest that the same set of factors do not interact with all of the proximal class II X box elements to mediate transcription.

MATERIALS AND METHODS

Cell Culture and Nuclear Extract Preparation

Raji, a class II-positive Burkitt's lymphoma cell line, was grown in RPMI 1640 medium (GIBCO, Inc.), supplemented with 2% fetal bovine serum, 8% calf serum, 100 U/ml penicillin, 100 μ g/ml of streptomycin, and 2 mM L-glutamine. Nuclear extracts were prepared from 10−15 g of packed cells as described by Shapiro *et al.* (20). Fifteen mg of nuclear extract protein was chromatographed on an Affigel heparin agarose column (BioRad, Inc.) as described earlier (15,21).

Oligonucleotide Probe Preparation

X box containing oligonucleotides, DRAX, DRBX, DQAX, DQBX, DPBX, and a Y box containing oligonucleotide, DRAY, were synthesized on a Gene Assembler Plus Oligonucleotide Synthesizer (Pharmacia, Inc.). The sequence of each oligonucleotide is shown in Figure 1. Individual strands were purified and hybridized with their complements as described earlier (15). Double-stranded oligonucleotide probes were prepared by end-labeling the duplexes using [γ-^{32}P]ATP (Amersham, Inc.) and T4 polynucleotide kinase (BRL, Inc.) to a specific activity of approximately 2×10^8 cpm/μg. Labeled probes were purified on native polyacrylamide gels, eluted into a solution of 0.5 M ammonium acetate (pH 7.5) and 1 mM EDTA at 37°C, and concentrated on Nensorb 20 columns (DuPont, Inc.). Probes used for methylation interference assays were prepared by labeling the appropriate oligonucleotides with [γ-^{32}P]ATP and T4 polynucleotide kinase, heat inactivating the enzyme (80°C for 3 min.), and annealing the single-stranded probes to their unlabeled complementary strands. Duplexed DNAs were gel purified as above. The labeled DNAs were methylated with 1 μl dimethyl sulfate (Sigma, Inc.) in a 200 μl solution of 50 mM sodium cacodylate, 1 mM EDTA for 20 minutes at 20°C and then quenched with 40 μl 1 M β-mercaptoethanol, 1.5 M sodium acetate. Methylated DNAs were ethanol precipitated twice, washed with 70% ethanol, dried, and dissolved in 0.2 M KCl.

Electrophoretic Mobility Shift Assays (EMSAs) and Methylation Interference

DNA-protein complexes were analyzed by EMSA as described earlier (15) For methylation interference assays, the standard binding reaction was scaled up approximately ten-fold using 5×10^6 cpm of single-end labeled probe. The complexes were separated by native polyacrylamide gel electrophoresis, and the entire gel was electroblotted to DEAE cellulose membrane (NA45, Schleicher and Schuell Inc.) in a solution of 25 mM Tris-HCl, pH 8.3, 190 mM glycine, 1 mM EDTA at 200 mA for 2 hours. Prior to transfer, the DEAE membrane was treated with 10 mM EDTA for 10 minutes, 0.5 M NaOH for 5 minutes, followed by several washes with water as directed by the manufacturer. Following transfer, the membrane was dried briefly, and exposed to X ray film for 30−60 minutes. Bands were excised from the membrane and the DNA eluted from the slices in 1.5 M sodium acetate (pH 7.0), 1 mM EDTA. Following chloroform extraction, the DNA was ethanol precipitated. The methylated DNA was cleaved in a solution of 1 M piperidine, 20 mM ammonium acetate, and 0.1 mM EDTA for 15 minutes, and the solution was lyophilized three times with resuspension in water between lyophilizations. Samples were analyzed on a 10% DNA sequencing gel, using standard conditions (22). Autoradiography was carried out overnight with an intensifying screen at −70°C.

Western Blot

Protein samples were separated on an 8% SDS-PAGE (23). The gel was then electroblotted to nitrocellulose membrane in a solution of 12% methanol, 25 mM Tris-HCl, pH 9.0, and 190 mM glycine. The nitrocellulose had been pretreated in 0.5 M NaOH, washed several times in water, once in 0.1M Tris-HCl, pH 7.5, and stored in the same buffer at 4°C until use. Following transfer, membranes were blocked for one hour at 20° with TBST

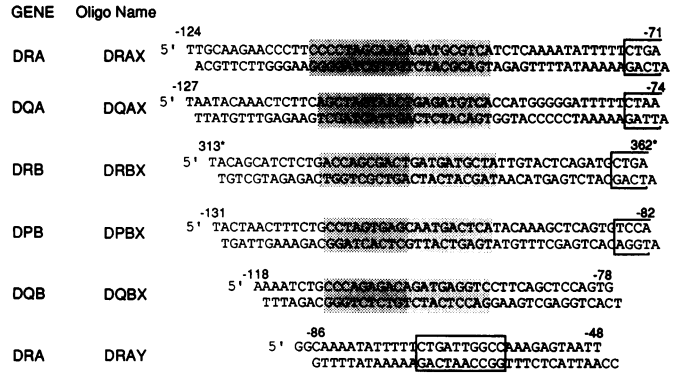

Figure 1. Oligonucleotides Used in this Study. Sequences of double-stranded X box oligonucleotides are shown for DRA (30), DQA (31), DRB (32), DPB (33), and DQB (34) genes. Basepair positions upstream from the transcriptional start site are indicated for all but DRB. The numbering for DRB is from Andersson *et al.* (32). The X1 and X2 boxes are shaded and the 5′-end of the Y box is indicated by brackets. An oligonucleotide containing the Y box of the DRA gene (DRAY) (outlined) used as a nonspecific competitor for X box binding analysis is shown.

(10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 1.5% gelatin and 1% horse serum. Membranes were washed three times with TBST and incubated with primary antibody at a concentration of 10 μg/ml. Polyclonal rabbit anti-c-Jun antibody (Oncogene Science, Inc.) recognizes a peptide corresponding to residues 73 to 87 in the N-terminal region of v-*jun*. This antibody detects c-Jun and cross-reacts with several other Jun-like proteins (Oncogene Sciences, Inc.). Rabbit anti-c-Fos antibody (Oncogene Science, Inc.) was raised against a peptide corresponding to amino acids 4 to 17 of human c-*fos*. Following three washes with TBST, filters were incubated for 30 minutes with a secondary donkey anti-rabbit antibody conjugated to horseradish peroxidase at a concentration of 0.2 μg/ml of TBST. Following five washes, filters were developed using the ECL (enhanced chemiluminescence) Western blotting detection system (Amersham Inc.) and exposed to X-ray film for 30 seconds.

RESULTS

Identification of Two Distinct X Box-Specific Complexes in B Cell Nuclear Extracts

To compare B cell factors binding to the X box region of the DRA gene with those recognizing the DRB X box, EMSAs were carried out on both crude and fractionated nuclear extracts prepared from Raji cells. Numerous complexes were detected when crude Raji extract was incubated with a probe containing the DRA X box sequence (DRAX) (Figure 2A, lane C). Fractionation of the extract on heparin agarose separated the complexes into two major peaks of X box-specific DNA binding activity (Figure 2A, fractions 32 to 41 and 47 to 56). The binding activity of the first peak (centered in fraction 38) was shown previously to have an identical binding pattern to mutant X box sequences as recombinant RFX protein and was thus identified as the native RFX activity found in our B cell extracts (15). The complexes that centered in fraction 53 migrated slightly faster through the gel than those of RFX and eluted from the heparin agarose column at approximately 0.43 M KCl. When an oligonucleotide containing the X box region of the DRB gene (DRBX) was used to probe crude Raji nuclear extracts, five specific complexes were formed as well as two faster migrating nonspecific complexes (Figure 2B, lane C). A similar analysis of Raji gradient fractions showed that these five specific complexes were found in fractions 47 through 56 (Figure 2B). In contrast to DRA X box sequences, the DRBX probe formed only weak specific complexes in fraction 38, suggesting that the DRBX oligonucleotide does not bind RFX tightly and that the binding activity in fractions 47 through 56 is distinct from RFX. Multiple specific complexes are observed in both the RFX fractions and in fractions 47 through 56. Within each of these specific activities, the complexes appear to have equivalent specificities and may represent different forms or multimers of the DNA/protein complex. It is unlikely that these multiple complexes are due to degradation of the nuclear proteins as this preparation, when assayed with a DRA Y box-containing probe (Figure 1), forms a single specific DNA/protein complex (data not shown).

Competition analysis of the two binding activities (RFX and that centered in fraction 53) showed that both are specific to the HLA-DRA X box and other class II X box regions; however, their binding activities differed in their relative affinities for each of these sequences. The RFX/DRAX complexes were readily competed away by increasing amounts of unlabeled DRAX oligonucleotide present in the binding reaction (Figure 3A). In contrast, a nonspecific competitor DNA (DRAY) containing the DRA Y box sequence failed to compete for these same proteins. In addition, complex formation showed different degrees of cross-specificity for X box sequences found in other human class II genes. In concordance with the analysis of Kobr *et al.* (16), the X box of the DQA gene competed weakly for RFX relative to competition with DRAX. All of the X box sequences from the β chain genes competed weakly for RFX. The DQB X box bound to RFX as tightly as that of the DQA gene, and competition with the DRB and DPB X boxes was detectable only with higher concentrations of competitor DNA.

The binding activity centered in fraction 53 showed a markedly different binding profile to the X box sequences of other human class II genes than that demonstrated by RFX. All five complexes formed with DRAX in fraction 53 were specific because they were all competed by an unlabeled DRAX competitor and not by the nonspecific oligonucleotide DRAY (Figure 3B). Further cross-competition assays revealed varying relative affinities to different class II gene X boxes. The X box sequence of the DRB gene competed for these same proteins nearly as well as that of the DRA gene, and the DPB gene X box DNA competed slightly

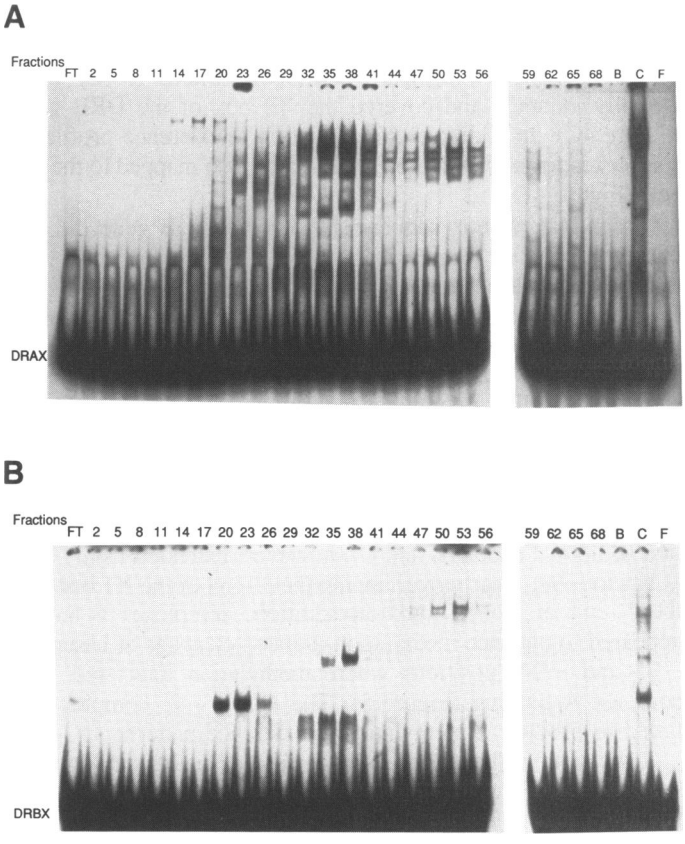

Figure 2. EMSA Analysis of Fractionated Raji Nuclear Extract. Autoradiographs showing EMSA analysis of Raji nuclear extract fractionated on a heparin agarose column. Approximately 0.15 μg of every third fraction was assayed in the presence of 200 ng of poly (dIdC)-(dIdC) and 50 ng of boiled salmon sperm DNA. (A) Gradient fractions probed with the DRAX DNA (Figure 1). Fraction numbers are as indicated above each well; C represents EMSA using 4 μg of crude Raji nuclear extract, 1 μg poly (dIdC)-(dIdC) and 0.5 μg boiled salmon sperm DNA; FT, 4 μl flowthrough fraction; B represents sample taken from 0.75 M KCl wash of the column; F represents probe alone. (B) Gradient fractions probed with DRBX DNA (Figure 1). Wells are labeled as above.

better (Figure 3B). In contrast, increasing amounts of DQA X box DNA competed very weakly for these proteins. Both the DQB and DPA (data not shown) X box DNA did not compete at all. The converse experiment is shown in Figure 3C. The five complexes formed with the DRBX probe and fraction 53 were readily competed away by DRB, DPB, and DRA X box sequences. The DQA X-box DNA competed very poorly, and the DQB X-box DNA did not compete for binding.

Binding Activity to the X1 and X2 Regions

Methylation interference studies were used to define the basepairs contacted by the binding activities in fractions 38 and 53. The methylation interference pattern generated from fraction 38 maps to a region between −110 and −98 bp upstream of the transcriptional start site and does not include the X2 box (Figure 4A). This pattern, which includes a hypermethylated guanine at −108 is identical to that previously shown by Reith *et al.* for RFX (14). In contrast, when fraction 53 was incubated with a methylated DRAX oligonucleotide, none of the five X box-specific complexes (Figure 3B bands A−E) were able to form if the DNA was methylated at the guanines between −98 and −88 bp (Figure 4B), designated the X2 box. This factor was termed X2BP.

Using crude nuclear extracts, similar methylation interference profiles were generated with other human class II X box DNA sequences that formed specific complexes in the EMSAs shown above. The methylation interference patterns for all five X box-specific complexes formed with DRBX (bands A−E) were virtually identical and covered the X2 box of the DRB gene (Figure 4C). In addition, a methylation interference profile of complexes formed with the DPBX probe also mapped to the X2 box (Figure 4D).

In contrast, X box sequences from the DQB gene did not compete for X2BP (Figure 3); however, EMSAs performed with crude extracts detected binding activities for both the X1 and X2 boxes. EMSAs with crude Raji extract generated a slowly migrating complex (Figure 5 inset, Band A) that was competed strongly by DRAX, DPBX, and DRBX competitors (data not shown), as well as two higher mobility bands (Bands B and C). Formation of the uppermost complex (A) was interfered by methylation at the GC basepairs between −100 and −94, and thus covered an analogous region to that bound by X2BP (Figures 5 and 6). Methylation interference patterns produced from the two additional bands (B and C), however, extended from bases −104 to −97, clearly overlapping the 3′-end of the X1 box and the 5′-end of the X2 box. In addition, complexes B and C appeared to enhance methylation-induced cleavage at basepairs −95 and −94, positions where methylation interfered with complex A binding (Figure 5). The nonspecific complex (D) failed to produce a methylation interference pattern.

A summary of the methylation interference analysis in Figure 6 shows that X2BP protects the X2 region in the DRA, DRB, and DPB promoters. Using the DRA numbering system, guanines at −95 and −91 and an adenine at −97 are shared between the three binding sites. A weaker guanine methylation interference position at −98 is shared between DRA and DRB.

Although there are some overlapping positions, the methylation interference pattern for the DQB X box is different from that described by X2BP interference experiments for DRA, DRB, and DPB. Moreover, DQBX oligonucleotides failed to compete for X2BP binding. DQBX complex A (Figure 5), which extends the methylation interference pattern into the X2 region is therefore

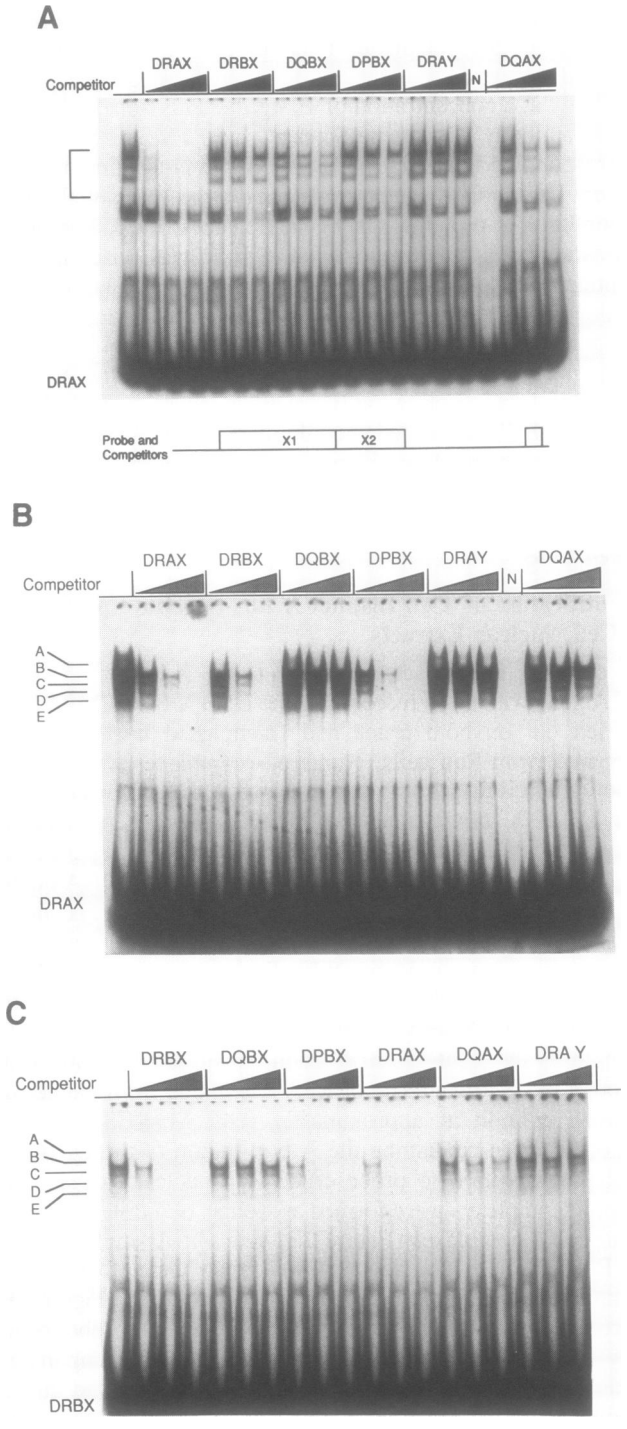

Figure 3. Cross-Competition Analysis of X Box-Specific Binding Activity in Fractionated Raji Nuclear Extract. Autoradiograph of EMSAs carried out in the presence or absence of unlabeled oligonucleotide competitors listed in Figure 1. Indicated protein fractions were preincubated with 200 ng of poly (dIdC)-(dIdC) and 50 ng of sonicated salmon sperm DNA plus or minus competitor. A graphic representation of the probes and competitors is shown. (A) Cross-competition analysis of DRAX-binding activity in heparin agarose Fraction 37 (RFX). 10, 50, and 100 ng of competitor oligonucleotide were added to the binding reactions at the preincubation step as indicated by the wedge above the wells. Bracket indicates RFX-specific binding activity. Lane N represents probe alone. (B) Cross-competition analysis of DRAX-binding activity in Fraction 53. Binding conditions were as indicated above. Complexes A through E were isolated for methylation interference analysis in Figure 4A. (C) Cross-competition analysis of DRBX binding activity in Fraction 53. Competition conditions are as indicated above. Individual bands (A−E) represent X box-specific complexes isolated for methylation interference assays in Figure 4B.

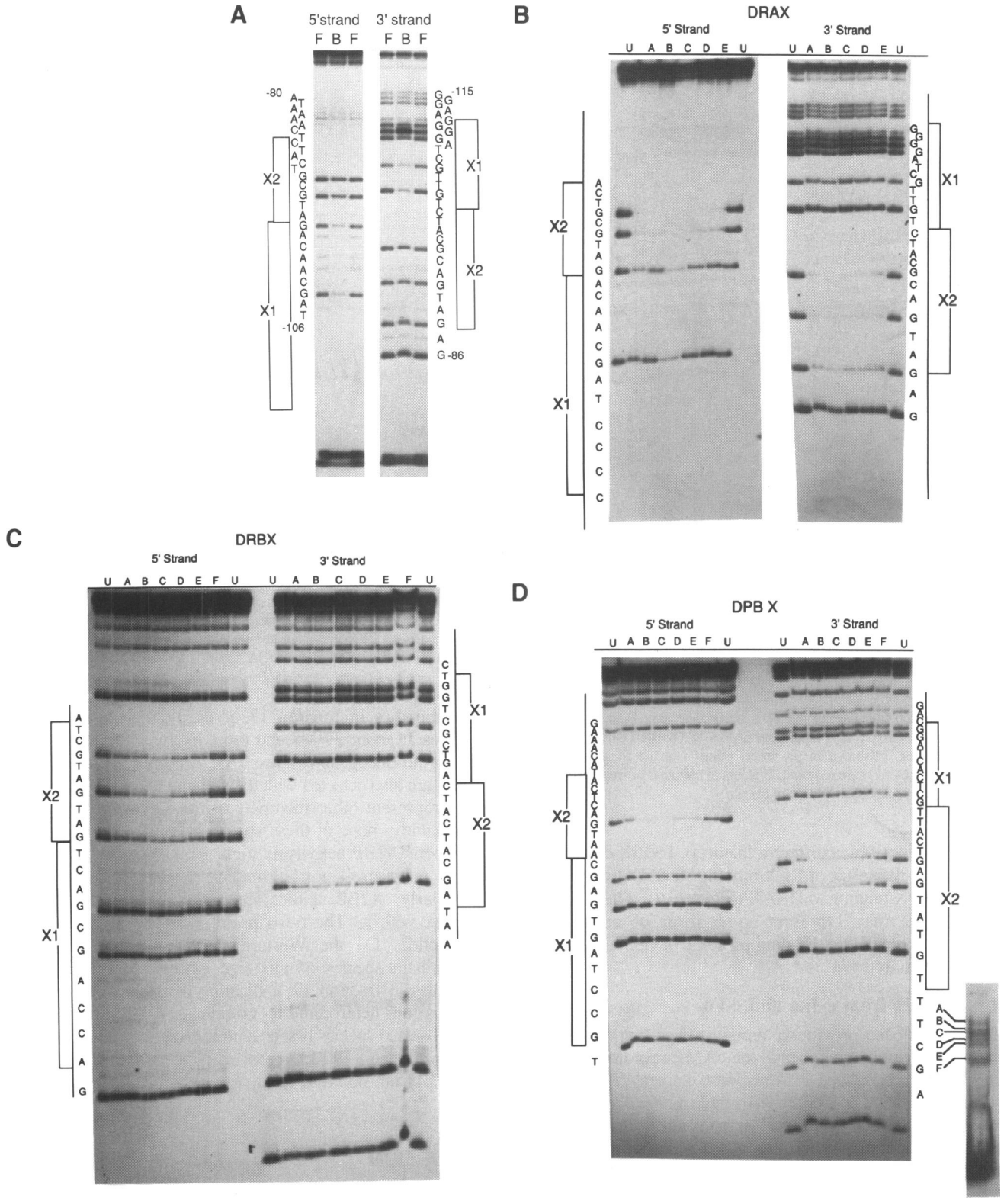

Figure 4. Methylation Interference Analysis of X1 and X2 Binding Activity. (A) Autoradiograph showing methylation interference analysis of DRAX probes binding X box-specific complexes in fraction 38. F and B are free and bound probe, respectively. The bound probe was obtained from the top band of a preparative EMSA similar to the one shown in Figure 3A. Schematic representation of the X1 and X2 boxes are indicated along the side of the figure as is the nucleotide sequence. (B) Methylation interference analysis of DRAX probes binding X box-specific activity centered in fraction 53. Lanes A–E were derived from complexes similar to those shown in Figure 3B. DRAX DNAs labeled at 5′- and 3′-ends are indicated. The lower intensity of some of the bands in lane C (5′ strand) are most likely due to less material loaded on the gel. Alternatively, this could mean that there is some RFX-like activity in complex C. U refers to unbound or free methylated DNA. (C) Methylation interference analysis of DRBX probes binding X box-specific complexes from crude Raji nuclear extract. X box-specific bands (A–E) are as denoted in Figure 3C. An additional higher mobility, nonspecific band, F, was isolated from EMSAs (not shown). (D) Methylation interference analysis of DPBX probes binding X box-specific complexes from crude Raji nuclear extract. X box-specific bands (A–E) as well as a nonspecific band (F) are denoted in the EMSA shown in the inset.

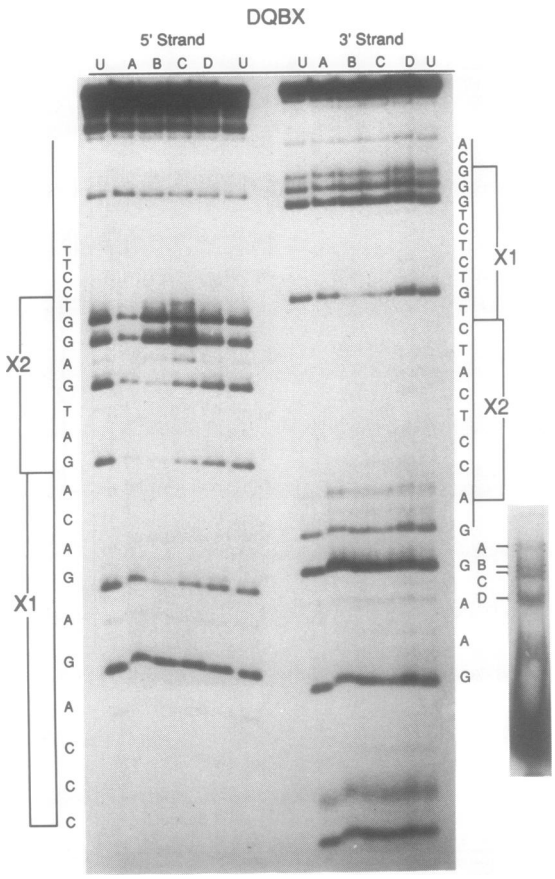

Figure 5. Methylation Interference Analysis of DQB X box Binding Activity in Crude Raji Nuclear Extracts. Preparative EMSA of DQBX DNA binding to crude nuclear extracts is shown in the inset. Bands isolated for methylation interference analysis (A–D) are as noted. U refers to unbound or free methylated DNA. Both 5′ and 3′-labeled DNAs are shown.

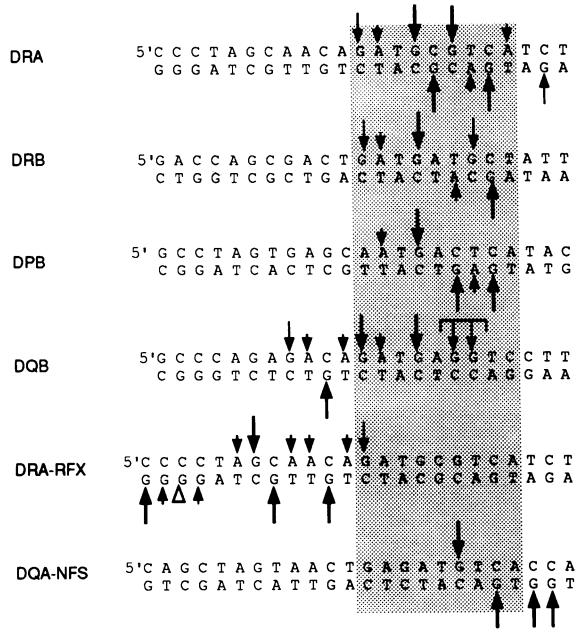

Figure 6. Summary of Methylation Interference Data. The X box regions of DRA, DRB, DPB, DQB, and DQA and DQB genes are shown. The X2 box is shaded. Large arrows denote bases where methylation strongly inhibited X2BP binding, and small arrows indicate weaker interactions. Bracketed arrow shown in the X2 region of the DQB gene represents positions that showed reduced methylation-induced cleavage in Band A or enhanced cleavage with Bands B and C (Figure 5). For comparison, RFX methylation interference on the DRA X1 box and NFS methylation interference on the DQA spacer region (16) are shown.

likely to be produced by a different factor(s). DQBX complexes B and C may be the result of RFX binding since DQBX weakly competes for RFX binding to DRAX (Figure 3A). Alternatively, these complexes may represent some form of cooperative interaction between X box binding proteins or define a new X box binding activity.

X2BP Is Distinct from c-Jun and c-Fos

The X2 box has been previously recognized as homologous to two other transcriptional elements, the cAMP response element (CRE) or the closely-related TPA response element (TRE). Liou *et al.* (24) used the X2 box of the I-Aα gene as a probe to isolate murine cDNA's from a λgt11 expression library. One of these, mXBP/CRE-BP2 belongs to a family of CRE binding proteins and complexes with the c-Jun protein via a leucine zipper dimerization motif (25). In contrast, the factor hXBP-1, isolated from a human cDNA library, complexes with c-Fos *in vitro* (18,19). In a similar manner, Andersson and Peterlin (12) used the DRA X2 box sequence to isolate a human cDNA that encodes c-Jun. Thus, it was plausible that X2BP was either AP-1 or complexes with c-Jun or c-Fos. To test this possibility, Western blots were performed on fractions taken from the heparin agarose gradient of the Raji nuclear extract and probed with antibodies generated against peptide epitopes contained in either c-Jun or c-Fos (Figure 7). The predominant species detected by the anti-Jun antibodies in fraction 17 of the heparin agarose column migrates at about 39 kD and most likely is the product of the c-jun proto-oncogene (Figure 7A). A number of cross-reactive species are also detected with the anti-Jun antibodies. These most likely represent other members of the Jun family of proteins. Significantly, none of these species were detectable in fraction 52, where X2BP activity is high. This suggests that X2BP is neither c-Jun itself nor is complexed with it.

Similarly, X2BP neither appears to be c-Fos nor forms a complex with it. The c-fos proto-oncogene encodes a 62 kD polypeptide. On the Western blot shown in Figure 7B, a predominant species of this size is detected by the anti-Fos antibodies in fraction 17, indicating that this fraction contains the Fos-Jun heterodimeric complex, AP-1. In addition, a similarly-sized species is seen eluting from the column at fraction 37 as well as a ~90 kD cross-reactive protein in fractions 37 and 42. Again, no cross-reactive activity can be seen in fraction 52.

DISCUSSION

These results demonstrated the existence of at least two different binding activities in B-cell extracts that interact with high affinity with the DRA X box region. This distinction was based upon their elution profile from a heparin agarose column, differential binding patterns to a variety of human class II X box sequences, and dissimilar DNA contact points within the X box region. The binding of RFX has been previously described by methylation interference and mutagenesis studies to occur in the X1 box of the DRA promoter (approx., −112 to −98 bp) (14,15). In DRA, the X2 binding protein, X2BP, binds immediately downstream

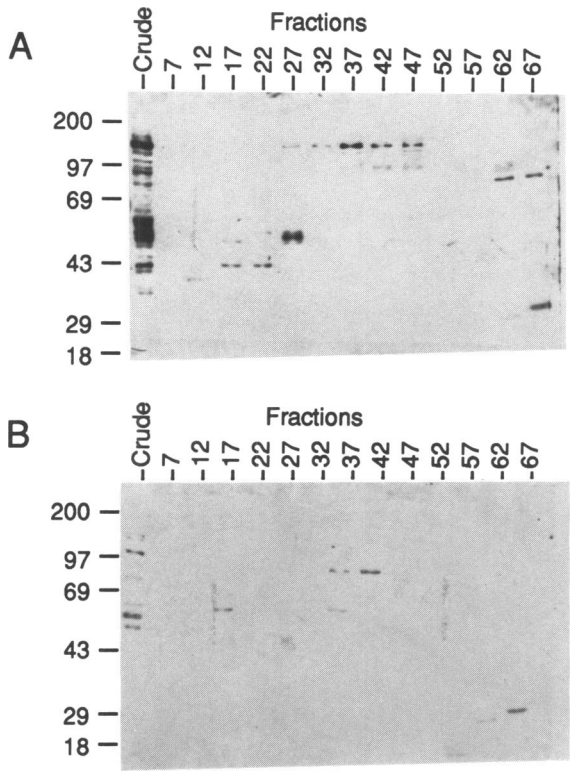

Figure 7. Immunoblot Analysis of Raji Fractions by Anti-c-Jun and Anti-c-Fos Antibodies. Autoradiograph of immunoblots detected by chemiluminescence are shown with heparin agarose fraction numbers as indicated above each well. Approximately 0.37μg of each fraction was loaded on the gel in the numbered lanes, and 8 μg of crude Raji nuclear extract was loaded onto the lane labeled C. Positions of molecular weight markers are as indicated along the side. (A) Immunoblot probed with anti-c-Jun antibody. (B) Immunoblot probed with anti-c-Fos antibody.

of RFX (−98 to −88 bp). RFX and X2BP showed different affinities for the different human class II gene X boxes. Extending the competition analysis of Kobr *et al.* (16), RFX binds to X box sequences with a relative affinity gradient of: DRA > DPA >> DQA = DQB and does not bind to DRB or DPB X boxes. In contrast, X2BP shows a relative affinity gradient of: DPB > DRA > DRB and does not bind to DQA, DQB or DPA X boxes.

Is X2BP the same as one of the previously described factors that interact with human class II X2 boxes such as HB16 (11), hXBP-1 (18), NF-X2 (c-Jun) (12), and NFS (16)? A comparison of X2BP binding activity to the binding properties of these other factors suggest that X2BP may be unique. Of all these other proteins, only hXBP-1 binds strongly to DRA X2 sequences and is found in human B cells. The factor hXBP-1, cloned by Liou *et al.*, binds to the DRA and DPB X2 boxes but not to homologous regions of the DRB, DQB, and DQA genes (18,19). In contrast, X2BP binds with a high affinity to the DRB X2 box. In addition, recombinant hXBP-1 was shown to form heterodimers with c-Fos *in vitro* (19). Our data do not indicate such an interaction with X2BP (Figure 7). Although it appears that X2BP may be distinct from hXBP-1, the possibility still exists that fractionation of the B-cell extract resulted in the dissociation of a hXBP-1/c-Fos heterodimer and that the additional binding activity attributed to X2BP may represent differences in assay conditions. If X2BP and hXBP-1 are distinct, these differences, which include length of oligonucleotide probe, salt concentration, and the temperature at which the reactions were performed (18), may also explain why our assays do not detect hXBP-1. Alternatively, hXBP-1 and X2BP may be part of a family of proteins that can interact with this sequence. In our EMSAs multiple complexes are formed whereas hXBP-1 forms a single complex with a shorter DRA X box probe (18). Each of our X2-specific complexes generate identical methylation interference patterns; thus, it is possible that one of the complexes represents hXBP-1.

A role for the X2 box in the regulation of class II gene expression in both class II-positive and γIFN-induced cells has been shown by linker-scanning mutagenesis studies (9,10,26). In addition, certain single basepair substitutions in the X2 box severely reduced the expression of DRA gene constructs in B cells (Sloan and Boss, unpublished data). Substitutions at basepair positions −98, −97, −94, and −93 showed the greatest transcriptional effects and correlate with the methylation interference data for X2BP binding activity. It is possible, therefore, that X2BP mediates this level of class II regulation.

It has long been assumed that the presence of conserved promoter elements indicated that a set of common factors could interact with all of the class II genes simultaneously to regulate transcription. The positive correlation between defective RFX binding to the DRA X1 box with a global class II transcriptional defect found in CID-cell lines (17) supported this model. In addition, the analysis of RFX binding activity to a series of point mutants in the DRA X1 box predicts that defective X1 box binding activity might be found in several other α chain gene promoters that show either low or absent transcriptional activity (15). Still, the lack of RFX binding to the X1 box of the highly transcribed class II gene DRB is puzzling and suggests that the 'common factor' model may prove too simplistic. However, all *in vitro* binding experiments have the caveat that the *in vivo* environment is more complex and that additional factors may stabilize low affinity DNA/protein interactions that are not detectable *in vitro*.

The evidence for multiple X2 binding factors (12,16,24) suggests that the existence of other RFX-like factors may also be possible, and that the presence of all of these factors may be essential for B cell-specific expression. Indeed, the existence of RFX mRNA transcripts in CID cells (27) suggests that RFX binding activity may be dependent upon yet another level of control, possibly post-translational. If other RFX-like factors exist, they may be likewise dependent upon modification for binding activity, and a lesion at this step may account for the CID. Alternatively, if an activated form of RFX serves as the common regulatory factor for all class II genes, other, as yet unidentified, X1 box motifs may prove to be important in class II expression of certain genes whose proximal X1 box element bind RFX poorly. Upstream elements, termed X′ and Y′ boxes, have been found greater than 1.1 kb upstream of the transcriptional start site of the murine I-Eα gene (28,29). These upstream sequences were necessary for proper I-Eα expression in I-Eα transgenic mice (13,29) and were proposed to participate in class II expression through a looping mechanism (28). If such elements are found in human genes, they may allow distally bound forms of RFX and/or X2BP to participate in transcriptional activation by overcoming low affinity interactions at proximal promoter elements. Thus, despite the current discrepancy between RFX binding activity and DRB transcription, a common set of factors and elements could still function coordinately to regulate class II expression.

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